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Preparation and characterization of crosslinked chitosan/gelatin scaffolds by ice segregation induced self-assembly

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ABSTRACT

Chitosan and gelatin are biodegradable and biocompatible polymers which may be used in the preparation of 3D scaffolds with applications in biomedicine. Chitosan/gelatin scaffolds crosslinked with glutaraldehyde were prepared by ice segregation induced self-assembly (ISISA); a unidirectional freezing at −196 °C followed freeze-drying to produce macroporous materials with a well-patterned structure. This process may be included within the green chemistry by the preparation of the porous structures without using organic solvents, moreover is a versatile, non-difficult and cheap process.

The scaffolds prepared by ISISA were characterized by scanning electron microscopy, attenuated total reflectance Fourier transform infrared spectroscopy, thermal gravimetric analysis, differential scanning calorimetry, and their stability was evaluated by degree swelling and degradation tests. The scaffolds present properties as high porosity, high degree swelling and good stability which make them suitable of applications as biomaterials.

1. Introduction

Hydrogels are three-dimensional, cross-linked polymeric networks which can swell in water forming soft elastic materials, and retain a significant fraction of water in their structure without dissolving (Hoffman, 2012; Peppas, Bures, Leobandung, & Ichikawa, 2000).

Hydrogels generate a great attention for their multiple applications and since they were introduced in the field of biomedicine (Wichterle & Lim, 1960), have been shown to have great potential as biomaterials (Guvendiren, Hoang, & Burdick, 2012; Lee & Mooney, 2001; Peppas, Hilt, Khademhosseini, & Langer, 2006), including their use as drugs delivery systems due to their ability to retaining a wide range of bioactive compounds (Contri, Soares, Pohlmann, & Guterres, 2014; Hamidi, Azadi, & Rafiei, 2008), in the development of materials for tissue regeneration (Balakrishnan et al., 2011; Hopkins et al., 2013; Slaughter, Khurshid, Fisher, Khademhosseini, & Peppas, 2009) or wound care (Pal, Banthia, & Majundar, 2007) thanks to their high biocompatibility, biodegradability and non-toxicity.

These features are because the physical properties of the hydrogels resemble those of living tissues more than any other kind of synthetic biomaterials, particularly in regard to their relatively high water content, soft and elastic consistency and low surface tension (Ratner & Hoffman, 1976). It is understandable that the hydrogels are proving to be particularly suitable as scaffolds in tissue engineering because they are able to mimic the three-dimensional environment of the cells in soft tissues. However, there are problems related to the hydrogels and are related largely to the macroscopic properties of the scaffold, particularly those associated with stiffness, strength, permeability, degradation rate and anchoring system. A suitable design of these variables is necessary to achieve a faster recovery and better quality of the regenerated tissue.

Chitosan [(1 → 4)-2-amino-2-deoxy-D-glucose] is a natural, linear and cationic polysaccharide obtained by deacetylation of chitin, the main component of the exoskeleton of crustaceans and insects and second most abundant polysaccharide in nature after cellulose (Kumar, 2000; Muzzarelli et al., 2012; Yeul & Rayalu, 2013). It is composed of structural units of 2-acetamido-2-deoxy-β-D-glucose (A-units) and 2-amino-2-deoxy-β-D-glucose (D units) linked by β-(1 → 4) distributed randomly along the chain.

Chitosan is insoluble in water, alkaline solutions and in organic solvents, however it can be dissolved in acid solutions below pH < 6.0, because amino groups (—NH_2) of glucosamine are protonated and enable the solubility of the molecule (Kurita, Kaji, Mori,

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& Nishiyama, 2000; Pillai, Paul, & Sharma, 2009; Rinaudo, 2006). Chitosan is considered as a structural analog of the glycosaminoglycans (GAGs) which are anionic polysaccharides present in the extracellular matrix of human tissues. GAGs are covalently linked to a core protein forming proteoglycans, which play an important role in the organization and function of the extracellular matrix (Gandhi et al., 2008; Lopez-Perez, da Silva, Serra, Pashkuleva, & Reis, 2010; Rigozzi, Müller, Stemmer, & Snedeker, 2013).

It is widely studied that chitosan is non-toxic, biodegradable, biocompatible, antimicrobial agent and has a hydrophilic surface that promotes cell adhesion and proliferation (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Rinaudo, 2008) which makes it an ideal candidate to use as biomaterial (Avetta et al., 2014; Muzzarelli, 2009, 2011; Shoichet, Kim, & Tator, 2011). Moreover when chitosan is crosslinked and/or mixed with others polymers its behavior in water and physical-chemistry properties may improves considerably (Garcia, Pinotti, & Zaritzky, 2006; Huang, Onyeri, Siewe, Moshfeghian, & Sundararajan, 2005; Monteiro & Airoldi, 1999).

Gelatin is a water soluble protein obtained from denaturation of collagen, which is the main structural protein of many connective tissues such as skin, tendon and bone. Similarly to chitosan gelatin is used as biomaterial due to its biocompatibility and biodegradability (Choi et al., 2001; Wu et al., 2010). Moreover, it has a low immunogenicity and may facilitate adhesion, cell proliferation and differentiation, because its composition contains arginine-glycine-aspartic acid, the amino acid sequence locates in the adhesion proteins of the extracellular matrix mainly in fibronectin, which interact with integrins at the cell membrane, facilitate adhesion of cells and biological responses triggered (Hersel, Dahmen, & Kessler, 2003).

Gelatin exhibits poor mechanical properties (Cheng et al., 2003) and is dissolved quickly in aqueous solutions, which is a drawback for its use as biomaterial. In the last years, several works showed that the mechanical properties and stability of gelatin may be improved using crosslinkers or blending with other polymers (Chiono et al., 2008; Kuijpers et al., 2000; Sarem, Moztarzadeh, & Mozafari, 2013). Moreover chitosan and gelatin used together improve the mechanical properties of the scaffolds prepared in comparison with those from single components (Tseng, Tsou, Wang, & Hsu, 2013).

Chitosan/gelatin scaffolds were obtained by ice segregation induced self-assembly (ISISA), a versatile, inexpensive technique and environmentally friendly used for the fabrication of porous scaffolds with different sizes, compositions and shapes. ISISA is a cryogenic process based on the unidirectional freezing of a hydrogel, aqueous suspensions or solutions into liquid nitrogen bath (−196 °C) at constant rate and subsequent freeze-drying. Ice crystals grow parallel to the freezing direction and solute particles dispersed in aqueous solutions are exuded to the boundaries between adjacent crystals of ice. After freeze-drying the ice is removed producing porous materials in which a porous network is generated corresponding to the empty areas where ice crystals were initially formed (Deville, Saiz, Nalla, & Tomsia, 2006; Mukai, Nishihara, & Tamon, 2003; Suwanchawalit, Patil, Kumar, Wongnawa, & Mann, 2009).

In the last years, various studies have demonstrated the ability of a cryogenic process using the ice crystals as a mold to control the morphology of the resultant porous structures by a unidirectional frozen process at a controlled immersion speed (Gutiérrez, García-Carvajal, Jobbágy, Rubio, et al., 2007; Mukai, Nishihara, Yoshida, Taniguchi, & Tamon, 2005). Furthermore, and considering the stability of many biological components in aqueous solutions, hierarchically organized structures have been prepared by implementing the modeling process by ice on a silica hydrogel containing proteins, liposomes or immobilizing bacteria and enzymes in the macroporous structure of a polymer cryogel (Ferrer, Esquembre, Ortega, Mateo, & del Monte, 2006; Gutiérrez, Jobbágy, Rapún, Ferrer, & del Monte, 2006; Gutiérrez, García-Carvajal, Jobbágy, Yuste, et al., 2007; Nieto et al., 2010).

In this work chitosan/gelatin scaffolds are produced with different weight ratios crosslinked with glutaraldehyde by ISISA process to find out the best composition for their use as a biomaterial. Physico-chemical properties of mixed scaffolds were studied by scanning electron microscopy (SEM), attenuated total reflectance-Fourier transform infrared (ATR-FTIR), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA). Enzymatic degradation and swelling tests were studied in physiological conditions to evaluate the efficacy of crosslinking.

2. Experimental

2.1. Materials

Low molecular weight chitosan (batch MKBH7256V, with 89% deacetylation degree (DD) and average molecular weight 558 kDa), gelatin (batch SLBB1129V, from bovine skin), glutaraldehyde (25% in water), lysozyme chicken egg white (batch BCBJ2814V) and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich Chemical Co. (Milwauke, WI, USA) and were used without further purification. Other chemicals were of analytical or spectroscopic reagent grade. MilliQ water ($R > 18\ M\Omega\ cm$) was used for the preparation of all aqueous solutions. All other chemical compounds were used as received.

2.2. Hydrogels preparation

Chitosan solution 2% was prepared in 0.2 M aqueous acetic acid at room temperature, left overnight on a magnetic stirrer. Gelatin solution 10% was prepared in deionized water at 40 °C.

To prepare mixed hydrogels, CHG, aqueous solutions of gelatin (10%) and chitosan (2%) were mixed with different weight ratios in a total volume of 1.5 ml. Each mixture was stirred at room temperature during 10 minutes to obtain a homogeneous solution and then 1% (w/w) of glutaraldehyde was added as crosslinker. The solution was vortexed for homogenization and placed in an insulin syringe and stored 3 h at room temperature for gelation.

After gelation the hydrogels were unidirectional frozen by dipping at a constant rate of 9.2 mm/min into liquid nitrogen bath maintained at a constant temperature of −196 °C. The frozen samples were freeze-dried using a ThermoSavant Modulyo-D freeze-drier (Thermo Electron Corporation, Waltham, MA, USA). The resulting dried hydrogels are called *scaffolds* or *cryogels* and kept the shape and the size of the insulin syringes.

Hydrogels with glutaraldehyde were called CHGAX, where CH is chitosan, G is gelatin, A is glutaraldehyde and *X* is the percentage of CH per sample (i.e. CHGA100).

CHG hydrogels without glutaraldehyde (called CHGX) were also prepared to evaluate the effect of the crosslinker.

2.3. Microstructural analysis

The morphology of the scaffolds was evaluated using field emission scanning electron microscopy (FESEM, Zeiss Ultra-Plus). The samples were fixed with conductive adhesive on aluminum support and sputter-coated with gold. They were observed at an accelerating voltage of 20 kV. The average cross section area of the pores was calculated using image-processing tools of the ImageJ software.